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(54) Title: GENE IDENTIFICATION METHOD

(57) Abstract

A method for the identification of genes that are essential for the maintenance of specific cell phenotypes is disclosed. The method includes the initial step of identifying a cell type with a phenotype of interest. Gene inactivation is performed on an aliquot of cells of the cell type of interest. Positive selection is then performed to an aliquot of the cell culture to which gene inactivation has been applied. Cells which continue to maintain the phenotype following gene inactivation have not had the gene of interest inactivated whereas cells in which genes necessary for maintaining the phenotype have been inactivated have been lost. Utilizing subtraction analysis between treated and untreated aliquots the gene in the cells which has been inactivated that affects the phenotype of interest is identified. Genes that are identified by the method are also disclosed as well as antibodies directed against the gene product of the identified genes. Further a customized kit for the practice of the gene identification method is also disclosed.

GENE IDENTIFICATION METHOD

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BACKGROUND OF THE INVENTION

5   1.   FIELD OF THE INVENTION

The present invention relates to a method of
identifying genes, specifically genes that maintain
specific cell phenotypes.

10   2.   DESCRIPTION OF RELATED ART

There are methods available to isolate and identify
specific genes. However these methods are not efficient
and rapid. Applicant has previous disclosed the
Technical Knock Out (TKO) selection method which has the
15   advantage of rapid isolation of genes that inhibit
proliferation in a specified restrictive environment
[Deiss and Kimchi, 1991; Deiss et al, 1995; Kissil et al,
1995; Deiss et al, 1996]. However, this method has the
limitation of requiring a phenotype that can be
20   efficiently selected against, such as a cell growth
arrest or cell killing phenotype.

Recently Smith et al [1995] and United States Patent
5,612,180 have described a method called genetic
footprinting to identify genes. The method involves
25   mutagenesis of potentially large numbers of genes
followed by a genetic selection of the cells containing
the mutated genes. This is followed by retrospective
analysis of the effect of individual gene inactivation on
the behavior cells containing these inactivations. From
30   this information new genes are determined. This method
has significant disadvantages for large scale gene
identification. The genetic footprinting method involves
mutagenesis by gene insertion and because of this
requires a haploid target which imposes a limitation on
35   the method. Second, the method of determining the effect

of each gene inactivation on the fitness of the cells containing the mutation involves a PCR amplification of the target gene which requires prior knowledge of the nucleotide sequence of all the target genes that will be studied which limits the gene base which can be searched. It would be useful to have a method which does not require a haploid target and does not require a known sequence.

It would be useful to have a rapid method which can identify genes to be isolated that are essential for the maintenance of specific cell phenotypes where positive selection exists for the phenotypes. These identified genes are excellent targets for the development of pharmacological inhibitors which would also act clinically to inhibit the specific phenotype. In other words it would be useful to have a tool which can effectively identify pharmacological targets for inhibition of deleterious phenotypes.

SUMMARY OF THE INVENTION

According to the present invention, a method for the identification of genes that are essential for the maintenance of specific cell phenotypes is disclosed. The method includes the initial step of identifying a cell type with a phenotype of interest. The method allows the phenotype of interest to be phenotypes relating to growth, phenotypes relating to release of factors and phenotypes relating to other basic cell functions.

Gene inactivation is performed on an aliquot of cells of the cell type of interest. Possible methods of gene inactivation include Genetic Suppressor Element (GSE) inactivation, Random Homozygous Knock-Out (RHKO) inactivation, or Technical Knock Out (TKO) inactivation.

Positive selection is then performed on an aliquot of the cell culture to which gene inactivation has been applied. The positive selection includes manipulations that test the ability of cells to survive under specific

5   culture conditions, ability to express a specific factor, changes in cell structure, or differential gene expression.

Cells which continue to maintain the phenotype following gene inactivation have not had the gene of

10   interest inactivated whereas cells in which genes necessary for maintaining the phenotype have been inactivated have been lost. Utilizing subtraction analysis between treated and untreated aliquots the gene in the cells which has been inactivated that affects the

15   phenotype of interest is identified. The subtraction analysis can include the methods of differential display, representational differential analysis (RDA), suppressive subtraction hybridization (SSH), serial analysis of gene expression (SAGE), gene expression microarray (GEM),

20   nucleic acid chip technology, or direct sequencing.

The invention further discloses the genes that are identified by the method of the present invention and for antibodies directed against the gene product of these identified genes. The present invention also provides

25   for a customized kit to practice the method of the present invention.

DESCRIPTION OF THE DRAWINGS

30   Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGURE 1 is a schematic representation of a general
outline of the method of the present invention; and
FIGURE 2 is a schematic representation of the method
of the present invention with a regulated anti-sense cDNA
5   expression library.

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, a method
10   for the identification of which genes are essential for
the maintenance of a specific cell phenotype is
disclosed. Phenotypes that can be studied are those for
which changes can be monitored in either haploid or
diploid cells. The method requires two general steps.
15   The first is the inactivation of genes in the cell by any
method known in the art and then in the second applying
positive selection for the phenotype of interest followed
by the identification via a subtraction analysis of the
gene in the cells which has been inactivated that affects
20   the phenotype of interest. By this method, a collection
of genes that are essential for the maintenance of a
specific phenotype are identified at the conclusion of
the procedure. The invention further discloses the genes
that are identified by the method of the present
25   invention and for antibodies directed against the gene
product of these identified genes.

Briefly, the method includes initially the
identification of a cell type for which genes controlling
its phenotype are needed. Once the cell type has been
30   identified, where required for the method an expression
cDNA library is constructed of the cells as they are
expressing the phenotype.

In general three methods are available for the gene
inactivation step: Genetic Suppressor Element (GSE)
35   [Holzmeyer et al, 1992; Roninson et al, 1995; Gudkov et

al, 1994], Random Homozygous Knock-Out (RHKO) [Li and
Cohen, 1996], and Technical Knock Out (TKO) described
herein above. Of these methods the TKO method is
preferred as it adaptable to the second step of the
5   present method as described herein below in Example 1.
Any method for gene inactivation may be used with
existing or later derived methods which can be adapted to
work with the second step of the present method is
preferred.

10   Following gene inactivation treatment, an aliquot of
the treated cells are exposed to a positive selection.
That is, the cells are exposed to conditions
requiring/activating the phenotype of interest. A
reserved aliquot of the treated cells is not exposed.
15   Following positive selection cells which continue to
express the desired phenotype remain and those cells
which cannot maintain the phenotype are lost. The method
then provides for determining the gene that was not
expressed in the lost cells by a "subtraction" analysis
20   by any method known in the art, generally utilizing a
comparison between the reserved cell aliquot and the
cells remaining after positive selection. It should be
noted that many aliquots can be tested and screened. The
gene(s) identified is at least one of the genes which
25   controls the phenotype.

The relative abundance of the differences between
the "targeted" and "untargeted" aliquots are
simultaneously compared using a "subtraction" analysis
(differential analysis) technique such as differential
30   display, representational difference analysis (RDA),
GEM-Gene Expression Microarrays (Schena et al., 1995;
Aiello et al., 1994; Shen et al., 1995; Bauer et al.,
1993; Liang and Pardee, 1992; 1995, Liang et al., 1993;
Braun et al., 1995, Hubank and Schatz, 1994; United

States Patent Number 5,545,531), suppressive subtraction hybridization (SSH) and direct sequencing (WO96/17957).

In the preferred method the procedure involves the transfection of targets cells with an anti-sense expression library followed by the positive selection of cells which have maintained a specific phenotype in the face of a specific challenge to the phenotype. It should be noted that one construct can be tested or many can be tested simultaneously in this method including over 100,000 constructs from an expression library. Cells in which an anti-sense inactivation has targeted a "sense" gene essential for the selected phenotype will be lost during the selection. Applicants have found that in general one cell has incorporated only one construct.

In this embodiment the next steps are to identify and isolate anti-sense expression vectors that are lost from the cell population due to cell loss during positive selection, that is, that induce a disadvantage in transfected cells during specific, positive selection resulting in the loss of the cell carrying the vector. These vectors are identified by subtracting the anti-sense expression vectors present after the selection from those present before the selection utilizing the reserved cell aliquot. This difference represents the vectors that express anti-sense against gene(s) that are essential for the maintenance of the selected phenotype.

These vectors are then recloned and sequenced. The identified anti-sense expression vectors are re-tested individually for the ability to inactivate the specific phenotype. With the sequence identified, the sense gene controlling the phenotype of interest can be identified using standard methods known in the art.

More specifically, the first part of the method consists of transfecting a target cell culture aliquot with an anti-sense expression library. The library is

generated by cloning a cDNA library in the anti-sense
orientation into an expression cassette that will express
the anti-sense strand at a high efficiency. The cassette
also contains a resistance marker that allows for
5   selection of cells that have been successfully
transfected. The cells that are transfected are ones
that express a phenotype of interest.

The transfection results in a pool of cells that
will express anti-sense messages against a large number
10 of the genes expressed in the cell. These anti-sense
messages will inactivate the functional expression of the
corresponding sense message. This results in a pool of
cells "knocked out" for the expression of many different
genes. In many cases due to the vector system used,
15 applicant have noted that the resulting cells will
contain only a single anti-sense expressing vector.

When a transfected cell looses a specific phenotype,
the anti-sense identity of the sense gene that has been
knocked out is identified by isolating and sequencing the
20 anti-sense expression cassette in the reserved unselected
(untreated) aliquot. The anti-sense strand on the anti-
sense expression cassette is the compliment of the sense
gene. If the anti-sense strand in not a full length anti-
sense, or does not match a sequence of a known gene, then
25 the gene fragment can be used as a hybridization probe in
order to isolate the full length gene. In essence, the
anti-sense expression vector serves as a tag to identify
the gene inactivation event of interest.

The method involves the selection of the pool of
30 anti-sense expressing cells for the specific phenotype.
The goal of the selection is to separate the majority of
cells which continue to maintain a specific phenotype
from the rare cells in which an anti-sense inactivation
event has specifically knocked-out a gene that is
35 essential for the maintenance of the specific phenotype.

This can be based on virtually any kind of positive selection means. These selection means can be varied as is known to those skilled in the art. However, the following is a non-exhaustive list and is not to be construed as limiting the present invention to these listed means.

The selection means is based on the ability of the cells to:

1. <u>Grow or survive under specific culture conditions, that is the actual selection is for the growth or survival of the cells</u>. In an embodiment, this can be basic culture conditions, such that the selection is for growth or survival-essential genes. The selection conditions could include sub-effective doses of specific factors which at effective doses would cause growth arrest or cell killing. In this case the selection is for the identification of knock-outs which sensitize the cells to the specific added factor.

In another embodiment, the selection can be in combination with a factor that normally does not cause an arrest or killing function. In this case a knock-out could be selected which only in combination with the added factor are effective in arresting or killing cells.

In a further embodiment, the selection can be for the inability to grow or survive when a parasite or infectious agent is added to the cell of interest. In this case the selection would be for knock-outs that are targeting genes that are specifically essential for some aspect of viral or parasitic function within a cell that are only essential when that cell is infected. Since some viral infection result in the induction of survival factors (such as CrmA, p35) it is likely that at least some cell functions are different and potentially selectively needed during viral, parasite growth.

2. _The second type of selection means is for the_
expression of a specific factor that can be measured and
this measurement can be adapted for a selection. This
factor can be anything that is accessible to measurement,
5   including but not limited to, secreted molecules, cell
surface molecules, soluble and insoluble molecules,
binding activities, activities that induce activities on
other cells or induce other organic or inorganic chemical
reactions.

10   3. _The third type of selection means is for changes_
in cell structure that are detected by any means that
could be adapted for a selection scheme. This includes,
but is not limited to, morphological changes that are
measured by physical methods such as differential
15   sedimentation, differential light scattering,
differential buoyant density, differential cell volume
selected by sieving.

         4. _The fourth type of selection means is based on_
differences in gene expression that can be directly
20   _measured_. This includes changes in cell surface markers,
changes in biochemical activities, any changes that would
be re-selected in changes in binding of fluorescent
labeled probes that could be used in conjunction with a
Fluorescence Activated Cell Sorter (FACS) or any property
25   that can be used as a basis for a selection.

        5. _The fifth type of selection means is based on_
differences in gene expression that can be indirectly
measured. This includes changes in transcription factor
activity that are measured by a synthetic gene construct
30   encoding a selective marker (such as a drug resistance
marker or a cell surface marker that could be used in a
FACS selection). This category would also include
changes in mRNA stability, mRNA localization, mRNA
translation control. All of these changes could be the
35   basis of a selection because a synthetic construct which

is controlled by one of these regulatory events could be
constructed which would drive the expression of an easily
selected gene product.

The third part of the method involves steps
identifying the anti-sense knock-outs that specifically
inhibit the phenotype of interest. Since the selection
of the anti-sense transfected cells is based on the
maintenance of the phenotype of interest, the cells of
interest (those loosing the phenotype) will not be
present after the selection but will be present before
the selection. Since the functional changes are caused
by expression from anti-sense expression vectors and the
inactivated genes can be identified by sequence analysis
of the cloned anti-sense cDNA insert, the goal of this
step is actually to identify the anti-sense expression
vectors that are lost from the population of cells during
the selection procedure.

The anti-sense inserts are cloned into a defined
position on the vector and the sequence elements
surrounding the site are known, so all the cDNA inserts
can be amplified with the use of a PCR amplification
using primers from the sequences that surround the insert
site. Thus the goal becomes to identify DNA molecules
present in one population and not in another. This is
accomplished by a variety of subtraction techniques.
Some of the methods that will be used are summarized
below as is known to those skilled in the art. However,
the following is a non-exhaustive list and is not to be
construed as limiting the present invention to these
listed means. Various differential hybridization methods
as well as different subtractive hybridization techniques
will be used. They are summarized in some detail in the
methods section.

Once fragments are identified that are lost during
the selection and are candidates for genes of interest

their function must be confirmed and the gene identified
in the fourth part of the method. The fragments will be
recloned into the anti-sense expression cassette and
individually re-transfected into the target cell to
5   determine whether the expression of the isolated fragment
can really change phenotype. If the phenotype is really
lost as is predicted then the isolated fragment will be
sequenced and used to isolate the full length sense gene.
It will also be determined whether the fragment is indeed
10   anti-sense with the use of strand specific probes. The
sense gene fragment will be used to derive antibodies
that can be used to monitor expression levels to
determine if there has been a functional anti-sense
knock-out [Deiss et al., 1995].

15   The present invention is a genetic method for
identifying genes that are essential for the maintenance
of specific cell phenotypes. The method requires that
the specific phenotype can be positively selected. These
identified genes are excellent targets for the
20   development of pharmacological inhibitors which would
also act clinically to inhibit the specific phenotype.
Thus the present invention provides a gene discovery tool
which can effectively identify pharmacological targets
for inhibition of deleterious phenotypes.

25   The following are several examples but this list is
not to be construed as limiting the present invention to
these listed examples.
Phenotypes related to growth or survival
        Addressing the problem of unusual growth: This
30   includes the problem of cancer but is not limited to
cancer but is applicable to all aberrant growth events.
The method of the present invention can be used to
identify genes that are essential for the growth of cells
transformed under general or specific conditions.

To define genes that are essential specifically for
transformed cells, an anti-sense cDNA library would be
introduced into a transformed and the non-transformed
cells that it was derived from. The anti-sense

5   constructs that interfere with transformed cell growth
and not from the non-transformed cells are found by
subtracting the anti-sense RNA molecules expressed in
surviving cells from both transfections. Knock-outs
specifically absent in the transformed cells but present

10   in the non-transformed cells are desired. These are
isolated by the methods described herein. The selection
can be a most specific selection such as one where sub-
lethal doses of chemotherapeutics are added during the
selection. In this case the selection would include gene

15   knock-outs that sensitize the cells to chemotherapeutic
treatments.

The factors added during the selection could be ones
that are thought to be present at the site of tumors.
Thus the selection would include events that sensitize

20   cells to a localized tumor effect and could increase the
specificity of anti-cancer treatment. Any growth or
survival event could be used as a basis not just cancer
related.

The growth or survival phenotype can also be used as

25   a way of eliminating populations of cells that are not
necessarily growing improperly but which function in a
manner that is deleterious. Thus virally infected cells
or parasite harboring cells could be used as a target and
the un-infected or non-parasite containing cells used to

30   subtract. This would define all the genes that are
specifically essential for the cell in the presence of
these insults. These would of course be excellent
targets for inhibiting viral or parasite spread.

35

Phenotypes related to the release of factors:

This class of selections includes events that
increase or decrease the production of secreted factors.
These include inflammatory mediators whose release could
be modulated. For example, if the production of a
specific mediator is necessary for normal immune function
but is produced at lethal levels in aberrant situations
(such as septic shock), then one could use the production
as a screen and look for events that knock-out or down-
regulate productions. In a further embodiment, the
selection can be done in the presence of sub-optimal
doses of other drugs in order to identify sensitization
events.

Phenotypes related to changes in cell functions:

These selection events are designed to identify
genes that are essential for many basic cell functions
that depend on any changes that can be externally
selected.

Several permutations of the method of the present
invention are possible and are presented as schematic
diagrams in Figures 1-2. Figure 1 provides a general
outline of the gene identification method of the present
invention. In this version a population of cells is
first transfected with an anti-sense cDNA expression
library. The expression library in this scheme codes for
a drug resistance marker that is used to select
transfected cells. This results in a population of cells
(Population 1) that all contain anti-sense expression
cassettes. The population of transfected cells is then
placed under a selection pressure. Cells that survive
this selection constitute population 2.

Transfected cells that become sensitive to the
selection procedure will be lost or at least reduced in
abundance in population 2. In order to identify the

constructs that induce this sensitization the following
procedure is performed. The expression cassettes
contained in the two population are extracted from the
cells. The cDNA inserts are excised by PCR amplification
5   using primers that flank the cDNA cloning sites. This
results in two pools of PCR fragments. To identify the
elements that are lost during the selection a subtraction
is done between the two pools. Elements are identified
that are present in population 1 and absent or reduced in
10 abundance in population 2.

To confirm that the subtracted fragments do indeed
induce a sensitization to the selection procedure,
individual fragments are recloned into the identical
vector and than individually retransfected into cells.
15 These cells are then individually assayed for sensitivity
to the selection procedure. A correctly cloned element
will induce sensitization of the transfected clones to
the selection procedure.

Figure 2 provides a diagram of the method with a
20 regulated anti-sense cDNA expression library. In this
simple variation the object is to clone the anti-sense
cDNA library into a vector in which expression of the
anti-sense is regulatable. The method is then modified
so that during the original transfection, the expression
25 of anti-sense is turned "OFF". After cells are selected
for the presence of the vector an aliquot of cells is
harvested and vectors are extracted and inserts excised
by PCR. This constitutes pool 1. The remaining
transfected cells are treated to turn "ON" the expression
30 of the anti-sense expression. An aliquot of these cells
are taken after several cell divisions (pool 2). Again
the aliquot of cells are extracted and cDNA inserts
excised by PCR. Finally an aliquot of the cells with
anti-sense turned "ON" is placed under a specific
35 selection and cells after this selection are harvested.

Again following extraction and PCR amplification we have
pool 3.

In this case we can perform two kinds of
subtractions. The first subtraction would be pool 2 from
pool 1. This identifies anti-sense inactivations that
are lethal or growth arresting. The second subtraction,
would be subtracting pool 3 from pool 2. This will
identify anti-sense knock-outs which sensitize cells to
the specific selection.

Additional permutations/variations on the method of
the present invention can be made. The method can be
used to identify different gene expression backgrounds.
In this variation anti-sense induced sensitization in
cells that express different genes is investigated. This
can be accomplished by transfecting into cells that
contain an inducible gene expression cassette. This
cassette affords inducible expression of a specific gene
construct we will call gene X for this example.
Following transfection and selection for the presence of
an anti-sense cDNA library and aliquot of cells is
harvested, vector extracted and cDNA inserts excised by
PCR. This is pool 1. The remaining cells are induced to
express gene X. Allowing some time for expression, the
cells are harvested, vectors extracted and cDNA inserts
excised by PCR. This generates pool 2. The subtraction
of pool 2 from pool 1 yields inserts that specifically
sensitize cells to the expression of gene X.

In another variation the method of the present
invention is used with different cell types. This
variation involves transfecting two different cell types.
This could be cells of different genetic background or of
different tissue origins, or even from different
organisms. In the simple diagramed case two cell types
are transfected with the same anti-sense cDNA expression
library. The different cell types are propagated in

-15-

different containers. Transfected cells are then
selected for the presence of the library. The cells
containing the library are harvested, vectors extracted
and cDNA inserts are excised by PCR. For each cell type

5   a different pool is generated. The subtraction between
these pools, both pool 1 from 2 and pool 2 from 1
identify anti sense knockouts that are specifically
lethal or growth arresting to one cell type but not the
other.

10   In a further variation, the method of the present
invention is used for determining the fitness of specific
genes in a population. In all the versions described
above, populations of PCR fragments are generated which
potentially differ by some number of elements due to the

15   biological activity of those elements. The subtraction
of these pools is then used as a method to identify cDNA
fragments which have biological effects when expressed.
It is also possible to use the same pools to determine
whether an anti-sense construct directed against a

20   specific gene could confer some biological effect during
some sort of selection. Specifically in this simple
example, when two pools 1 and 2 have been generated for
the operation such as in the examples, there are a
variety of tools available to individually measure the

25   relative abundance of anti sense construct representing
specific cDNAs in pool 1 and pool 2. A variety of
methods are known for quantitating the abundance of DNA
molecules in different samples. Following is a non-
exhaustive list: Southern blot analysis either using a

30   fragment of the gene of interest as a probe against the
two pools; Quantitative PCR with specific primers
identifying the gene of interest; GEM analysis, using the
pools 1 and 2 as the probes and hybridizing against chips
of arrayed known genes. If the abundance of the anti-

35   sense construct significantly decreases after a selection

then it is likely that anti-sense has sensitized the
cells to that selection.

As shown in Example 2 and Table 2 herein below,
sequences of genes have been identified by the method of
5   the present invention (SEQ ID Nos:15-36). An antisense
construct of these sequences delivered to a cell reduces
a gene product (gene inactivation) and thereby provides
sensitization of the cells to anti-Fas antibodies. In a
preferred embodiment the sequences are SEQ ID
10   Nos:19,20,23,25,26,36. These antisense constructs can be
used therapeutically to sensitize the cells for antibody
therapy. Antisense therapeutic construct can be
delivered to the cells and can be rendered nuclease
resistant as is known in the art [Agrawal, 1996;
15   Calabretta, et al, 1996; Crooke, 1995; Felgner, 1997;
Gewirtz, 1993; Hanania, et al 1995; Lefebvre-
d'Hellencourt et al, 1995; Lev-Lehman et al., 1997; Loke
et al, 1989; Wagner et al., 1996; Wagner, 1994;
Radhakrishnan et al., 1990.]
20   The present invention also discloses novel gene
sequences as set forth in SEQ ID Nos:18,21,25 and 30-32.

The present invention also provides for a customized
kit to practice the method of the present invention.
The kit would be assembled to include at least an
25   expression cDNA library constructed for specified cells
as they are expressing the phenotype. Further a culture
of cells of the requested phenotype could also be
provided in the kit.

The above discussion provides a factual basis for
30   the method of identifying genes that are essential for
the maintenance of specific cell phenotypes. The methods
used are shown below and can be shown by the following
non-limiting examples and accompanying figures.

35

EXAMPLES

GENERAL METHODS:

General methods in molecular biology: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989).

Polymerase chain reaction (PCR) was carried out generally as in *PCR Protocols: A Guide To Methods And Applications*, Academic Press, San Diego, CA (1990). Reactions and manipulations involving other nucleic acid techniques, unless stated otherwise, were performed as generally described in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, and methodology as set forth in United States patents 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 and incorporated herein by reference.

Recombinant Protein Purification is undertaken as generally set forth in Marshak et al, "Strategies for Protein Purification and Characterization. A laboratory course manual." CSHL Press, 1996 unless otherwise specified.

Vectors are constructed containing the cDNA of the present invention by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences (see below in specific methods for a more detailed description). Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. Phagemids are a specific example of such beneficial vectors because they can be used either as plasmids or as bacteriophage vectors. Examples of other vectors include viruses such

as bacteriophages, baculoviruses and retroviruses, DNA
viruses, cosmids, plasmids, liposomes and other
recombination vectors. The vectors can also contain
elements for use in either procaryotic or eucaryotic host
5   systems. One of ordinary skill in the art will know
which host systems are compatible with a particular
vector.

The vectors are introduced into cells or tissues by
any one of a variety of known methods within the art
10   (calcium phosphate transfection; electroporation;
lipofection; protoplast fusion; polybrene transfection).
The host cell can be any eucaryotic and procaryotic
cells, which can be transformed with the vector and which
will support the production of the enzyme. Methods for
15   transformation can be found generally described in
Sambrook et al., *Molecular Cloning: A Laboratory Manual*,
Cold Springs Harbor Laboratory, New York (1992), in
Ausubel et al., *Current Protocols in Molecular Biology*,
John Wiley and Sons, Baltimore, Maryland (1989), Chang et
20   al., *Somatic Gene Therapy*, CRC Press, Ann Arbor, MI
(1995), Vega et al., *Gene Targeting*, CRC Press, Ann
Arbor, MI (1995) and Gilboa, et al. (1986) and include,
for example, stable or transient transfection,
lipofection, electroporation and infection with
25   recombinant viral vectors. In addition, see United
States patent 4,866,042 for vectors involving the central
nervous system and also United States patents 5,464,764
and 5,487,992 for positive-negative selection methods.

General methods in immunology: Standard methods in
30   immunology known in the art and not specifically
described were generally followed as in Stites et
al.(eds), Basic and Clinical Immunology (8th Edition),
Appleton & Lange, Norwalk, CT (1994) and Mishell and
Shiigi (eds), Selected Methods in Cellular Immunology,
35   W.H. Freeman and Co., New York (1980).

Immunoassays: In general, ELISAs are the preferred
immunoassays employed to assess a specimen. ELISA assays
are well known to those skilled in the art. Both
polyclonal and monoclonal antibodies can be used in the
5   assays. Where appropriate other immunoassays, such as
radioimmunoassays (RIA) can be used as are known to those
in the art. Available immunoassays are extensively
described in the patent and scientific literature. See,
for example, United States patents 3,791,932; 3,839,153;
10   3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262;
3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074;
4,098,876; 4,879,219; 5,011,771 and 5,281,521 as well as
Sambrook et al, *Molecular Cloning: A Laboratory Manual*,
Cold Springs Harbor, New York, 1989.

15

Polyclonal and Monoclonal Antibody Production

Antibody Production: Antibodies may be either
monoclonal or polyclonal. Conveniently, the antibodies
may be prepared against a synthetic peptide based on the
20   sequence, or prepared recombinantly by cloning techniques
or the natural gene product and/or portions thereof may
be isolated and used as the immunogen. Such proteins or
peptides can be used to produce antibodies by standard
antibody production technology well known to those
25   skilled in the art as described generally in Harlow and
Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor
Laboratory, Cold Spring Harbor, NY, 1988 and Borrebaeck,
Antibody Engineering - A Practical Guide, W.H. Freeman
and Co., 1992.

30   For producing polyclonal antibodies a host, such as
a rabbit or goat, is immunized with the protein or
peptide, generally with an adjuvant and, if necessary,
coupled to a carrier; antibodies to the protein are
collected from the sera.

For producing monoclonal antibodies the technique
involves hyperimmunization of an appropriate donor with
the protein or peptide fragment, generally a mouse, and
isolation of splenic antibody producing cells. These

5   cells are fused to a cell having immortality, such as a
myeloma cell, to provide a fused cell hybrid which has
immortality and secretes the required antibody. The
cells are then cultured, in bulk, and the monoclonal
antibodies harvested from the culture media for use.

10   The antibody can be bound to a solid support
substrate or conjugated with a detectable moiety or be
both bound and conjugated as is well known in the art.
(For a general discussion of conjugation of fluorescent
or enzymatic moieties see Johnstone & Thorpe,

15   *Immunochemistry in Practice*, Blackwell Scientific
Publications, Oxford, 1982.) The binding of antibodies
to a solid support substrate is also well known in the
art. (see for a general discussion Harlow & Lane
Antibodies: A Laboratory Manual, Cold Spring Harbor

20   Laboratory Publications, New York, 1988 and Borrebaeck,
Antibody Engineering - A Practical Guide, W.H. Freeman
and Co., 1992) The detectable moieties contemplated with
the present invention can include, but are not limited
to, fluorescent, metallic, enzymatic and radioactive

25   markers such as biotin, gold, ferritin, alkaline
phosphatase, β-galactosidase, peroxidase, urease,
fluorescein, rhodamine, tritium, ^{14}C and iodination.

SPECIFIC METHODS

30   <u>Construction of anti-sense expression vector</u>:
This method is not limited to any specific vector
system. The actual requirements are that the vector
system express at high levels anti-sense molecules and
that they can be identified. In a preferred embodiment

the Epstein Barr Virus (EBV) Episomal vector is used
[Deiss et al, 1991].

The EBV episomal vector consists of DNA segments
that are necessary for the episomal maintenance of the
5   episome both in bacteria (*E. coli*) and in human cells
(this include an origin of replication and a trans-acting
factor (EBNA-1). The episome also includes genes
encoding resistance markers for selection either in
bacteria or in human cells. Finally the vector contains
10   a transcription cassette. Initially it will be based on
the vector as described in Deiss et al. [1991], but the
present invention contemplates any transcription cassette
that produces high levels of anti-sense expression. The
EBV episomal vector contains a RNA Polymerase II promoter
15   and or enhancer driving the transcription of a synthetic
transcript containing a set of cloning sites, a splice
donor and acceptor site and a polyadenylation signal,
followed by a second set of enhancers. This vector can
be efficiently shuttled from animal cells to bacteria and
20   vice versa. One procedure that allows for rapid
shuttling is using the method of Hirt [1967] to extract
episomal vectors from animal cells and using this
preparation to transform *E.coli*. Applicants have
observed that, on average, cells transfected with a
25   library cloned into the vector contain only one
expressing vector.

The specific choice of promoters and enhancers are
dependent on the exact selection condition and the cell
line used. This must be empirically determined for each
30   selection condition as is known to those skilled in the
art.

In an embodiment, the EBV vector can also contain an
inducible expressed promoter such that the expression of
the anti-sense library would be inducibly expressed by a

specific inducer.   This will allow additional flexibility in designing selection protocols.

<u>Construction of Anti-sense cDNA library</u>

There are several methods available to construct

5   directional cDNA libraries. Any of these methods would be sufficient since they result in the production of a directionally identified cDNA library and the practitioner can use the method they are most familiar with. The directional cDNA is then cloned into the

10   expression cassette in the anti-sense orientation. A method that may be used is detailed in Deiss et al, 1991. Briefly, it consists of making cDNA by the method of Gubler and Hoffman [1983] and making the cDNA directional by the method of Meisner et al. [1987].

15   The mRNA is extracted from cells that have been cultured under a variety of conditions that mimic the actual selection conditions. This is designed to ensure that the library will include all the messages that are expressed in the target cell under selection conditions.

20   RNA is prepared at time points that would contain messages that are always present as well as messages that are induced by the selection procedure. This is achieved by extracting RNA previous to the selection and at times during the selection. The various pools of RNA are then

25   mixed together so that all possible RNA molecules are present [Deiss and Kimchi, 1991].

An alternative method that can be used consists of deriving a library of genomic DNA fragments cloned into the expression cassette. Since all the transcribed

30   messages are derived from genomic DNA (with the exception of RNA edited messages; this will actually include mitochondrial DNA as well) this method would generate all possible messages. The directionality would be lost so the library would be only half anti-sense. Since the

35   sense fragments are unlikely to frequently encode full

length proteins or have biological activity the anti-sense fragments would still likely produce the most frequent biological effects. The genomic fragments are produced by restriction enzyme cleavage of genomic DNA.

5   Only one library per species would be necessary to produce, since with the exception of the B and T cell receptors, genomic DNA does not differ in different cell types at least in mammals (again erythrocytes or any cells that lack nuclei are an exception).

10   In the case of the genomic library it will be necessary to determine whether any expressed fragments express a sense or an anti-sense message. This is done by using the insert as a strand specific probe both from the expressed and non-expressed strand in a Northern

15 analysis. This indicates if the expressed fragment is sense or anti-sense in relation to the endogenously expressed gene.

         Some sequence will match, some will not match, genes already deposited in the various databases. In the case

20 where the identified gene matches the sequence of a gene already in a database this information would then enable the determination if the insert is a sense or anti-sense insert.

Transfection of the anti-sense library.

25   There are a large variety of methods to transfect DNA into cell lines and cell cultures. The most efficient method for each selection will be determined empirically based on experience and the known relative efficiency of each method.

30   The method selected must both efficiently delivery DNA into cells and not effect the biological responses that will be selected following the transfection. Viral vector system can also be used and this would entail producing infectious virus and infecting the target

35 cells. Applicant has found electroporation to be an

efficient method, but other methods can be used as are known in the art.

Identification of differentially expressed antisense messages

5   The methods of identifying the differentially expressed antisense messages in a preferred embodiment will include the methods described in Braun [1996] and as described in Diatchenko et al.[1996]. These methods include a PCR amplification of subtracted populations.

10   Appropriate restriction sites are included in the expression vector so that following PCR amplification of the cDNA inserts, the inserts are flanked by appropriate restriction sites. Restriction digestion is then used to produce templates that are useful for these techniques.

15   Another method that is used is the "GEM" gene expression microarray as described in Schena et al. [1995]. In this technique, PCR fragments corresponding to a set of specific plasmids (in this case antisense cDNA inserts contained in antisense expressing vectors or

20   other DNAs as appropriate) are fixed to a glass template and this is hybridized with two fluorescently labeled probes. In this specific case, the probes are reverse transcribed antisense transcripts derived from cells transfected with the antisense expression library either

25   before or following a selection.

Generation of efficient antisense inhibitor

In addition to assaying expression cassettes where all the transcripts are directionally cloned in the antisense orientation, another strategy employed in the

30   present invention is to generate randomly primed cDNA and cleave the cDNA with two restriction enzymes X and Y and clone the resulting mixture into two different expression cassette. In the first cassette site, X, would transcriptionally precede Y and the second cassette site,

35   Y, would transcriptionally precede X. In this

arrangement, the cDNA is divided into sections that may
have different abilities to serve as an efficient
antisense inhibitor. The strongest differential signal
is likely to be produced by the fragment that is the most
5   efficient antisense inhibitor. Thus, the screening is
more likely to produce a meaningful differential signal.

SPECIFIC EXAMPLES
DETERMINATION OF COMBINATION OF METHODS
10   FOR PREFERRED EMBODIMENT

The method requires two distinct major steps as
described herein above. It is greatly advantageous if
this method can be applied to a wide variety of cells.
It is therefore useful if both steps could be applied to
15   a wide variety of situations.

In the first step genes are inactivated in order to
determine whether individual genes are essential for a
specific phenotypic change. It is advantageous if these
inactivation will have a phenotype both in haploid cells
20   and in diploid cells. Since many cells of interest are
diploid in nature. Furthermore, it is also an advantage
if the inactivation method allows for the rapid
identification of the inactivated genes. This can be
achieved in a variety of manners. The inactivation
25   methods are generally based on one of three different
principles.

The first principle is that genes can be
functionally inactivated by expressing mRNA that is
derived from the anti-sense strand of the sense message.
30   This allows for inactivating the mRNA in the cell and
does not require a specific gene dose. This can work for
single copy or multiple copy genes either from haploid or
diploid organism. It has been shown that anti-sense
inactivation can be effective in a wide range of
35   organisms including bacterial, plant and animal.

Applicants and others have extended the original
observation by generating anti-sense expressing cDNA
libraries. Applicants termed this method Technical Knock
Out (TKO). These libraries contain collections of many
5   (usually 100,000 to 1,000,000) different anti-sense
expression constructs that will individually express a
single anti-sense RNA molecule when transfected into
appropriate target cells. Since these libraries contain
large collections of these vectors they in effect can
10   express anti-sense RNA to virtually all expressed mRNA
molecules. Several investigators have used these type of
libraries to inactivate genes and change the phenotype of
cells. Once an altered cell is identified the
expression cassette contained in the cells can be
15   identified since the expression cassette DNA sequence is
known. Subsequently, the anti-sense expressed cDNA
molecule that is contained in the expression cassette is
identified. This can be achieved by a variety of
methods; applicants have used two methods. The first
20   method involves shuttling the vector from animal cells
into bacterial cells. Once the vector is in bacterial
cells it is easy to produce large amounts of the vector
for further analysis. The second method we have employed
involves PCR amplification of the cDNA inserts by
25   designing PCR primers that flank the cDNA cloning site on
the vector. The flanking vector sequences are known so
it is easy to chose appropriate primers. PCR
amplification with these primers amplifies any cDNA
molecules that were present between the two primers. The
30   anti-sense approach also allows for tagging of the
inactivation event. That is the identity of the sense
message generally can be determined by sequencing the
anti sense construct. This construct can then be
identified and isolated from the phenotypically altered
35   cells.

The second gene inactivation method that fits these
requirement is an inactivation method that relies on
production of "dominant negative" fragments of genes from
an expression cDNA library. This method is called the
5   Genetic Suppressor Element method (GSE). It is based on
the observation that small fragments of a gene when
expressed may interfere with the normal function of the
full length gene product and in fact interfere with the
normal function. In this manner these gene fragments
10   were called "dominant negatives". A GSE library thus
consists of fragmented cDNA molecules which are cloned
into an expression cassette. When expressed from
translation initiation signals in the cDNA molecule or
from translation initiation signals present in the
15   expression cassette these gene fragments can interfere
with gene function. In addition the libraries used in
the GSE method also include some anti-sense fragments and
therefore gene inactivation can occur either by anti-
sense or by dominant negative inactivation of gene
20   function.

The third method of gene inactivation that could be
used is called "Random Homozygous Knock-Out (RHKO)". In
this method gene inactivation is achieved in two steps.
A retroviral vector is used to infect target cells. The
25.   integration of the retroviral element itself can lead to
inactivation of one copy of a gene if the integration
event itself functionally disrupts the normal
transcription or activity of the gene in which it
integrates. The retroviral vector used has an additional
30   property that it encodes a transcription element that
should transcribe into the chromosomal location in which
it has integrated. In the case that this generates and
anti-sense RNA transcript, additional copies of the gene
could be inactivated. Thus this method also relies on
35   anti-sense inactivation.

The TKO method was chosen for generating inactivations in these examples as the preferred embodiment because the other methods described above are not as compatible with the second step in the method of

5   the present invention as the TKO procedure. However, as improvements become available in these methods they could be used.

In the GSE method both sense and anti-sense gene fragments are generated which are expected to have

10 different biological activities. It is difficult to distinguish closely related gene fragments of this sort by the methods that will be used in the second part of the gene identification method of the present invention. Thus the rare molecules that cause biological changes

15 when expressed would be very difficult to distinguish from many similar molecules that would not have biological effects. The molecules that do not have effects would in essence mask the active molecules.

The RHKO method was also difficult to adapt to a

20 high throughput subtractive procedure. The potential anti- sense fragments generated in this procedure must be cloned out individually and this is a process that is hard to adapt to subtraction.

The TKO method was easily adapted to subtraction.

25 The cDNA inserts contained in expression vectors should be all or at least mostly anti-sense in nature. The cloning procedure outlined in Deiss and Kimchi was used. This generates anti-sense cDNA libraries and results in libraries that are biased to be anti-sense. It is

30 possible to obtain some sense cDNA inserts with this method. Thus since most of the fragments are antisense the subtraction step will be mainly between different cDNA fragments that were expressed as anti-sense constructs. Again the principle of the present invention

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30   possible to obtain some sense cDNA inserts with this
method. Thus since most of the fragments are antisense
the subtraction step will be mainly between different
cDNA fragments that were expressed as anti-sense
constructs. Again the principle of the present invention
35   is that the abundance of an anti-sense construct(s) that

induces a disadvantageous phenotype will be reduced after
a biological selection. We wish to identify these
constructs. Thus the TKO method was the method of choice
for the gene inactivation step of the present invention.

5   It can be used in a variety of cell populations, both in
haploid, diploid and aneuploid cells. It can be easily
scaled up to involve 100,000 events or more without undue
expense. And it can be easily adapted to the subtractive
methods that are needed in the second part of the method

10   of the present invention.

       The second step in the gene identification method
requires that we can identify the loss of specific anti-
sense gene constructs from a large population of anti-
sense constructs that are not lost. This can be

15   accomplished in a variety of different ways. Because it
is a great advantage to be able to identify specific
losses in the presence of large numbers of molecules that
are not lost we needed a method that has a high
throughput capacity. One method that fits this

20   requirement involves using high density arrayed chips
such as the GEM chips. These are arrayed dots containing
specific DNA molecules corresponding to genes. The dots
are arrayed at high density on a glass coverslip with the
position of each dot and the identity of the DNA molecule

25   fixed on each dot precisely determined. Two probes
derived from different population of DNA or RNA molecules
are labeled with two different fluorescent dyes and
hybridized to the arrays. After appropriate washing the
relative binding of the dyes at each dot is determined.

30   The amount of dye bound at each spot reflects the
abundance of the gene fixed on that dot relative in the
whole population. Thus when two populations of DNA
molecules are labeled with different dyes one can
accurately determine whether there has been a change in

35   relative abundance of individual molecules in the

population. If there is no change the ratio of the two
dyes will be one. If there has been a change in abundance
then the ratio of the two different dyes will also
change. This method can rapidly measure the changes of
5   large numbers of genes in a large population. A copy of
the DNA fix on each dot is stored and can be retrieved
for further analysis. Although in the following example
we did not use the GEM method to measure the loess of
anti-sense constructs it is a method that can be used in
10 the practice of the present invention.

A second method uses to identify the loss of
specific anti-sense gene constructs from a large
population of anti-sense constructs that are not lost is
called "Subtraction". This involves manipulating two
15 populations of DNA or RNA molecules so that only
molecules that are present in one population and not in
another are recovered. The version that we actually used
is called PCR-Select which is a commercially available
kit from CLONTECH. Briefly

20   1. Two populations of double stranded DNA are
generated. It is assumed that some of the dsDNA
molecules are present in only one of the populations (or
at least much more abundant in one population).

2. These populations are separately processed. The
25 population that is assumed to have extra species of
molecules is called the tester sample. The second
population that is assumed not to have these specific
species is called the driver. The tester population is
separately ligated to two different linkers. This
30 generates tester population 1 and tester population 2.
The driver is left without linkers.

3. A series of manipulations including denaturation
and renaturation of the driver and tester in various
combinations is used. This results in generating a
35 series of DNA molecules that have different set of

linkers at their ends. The only product that can be
effectively PCR amplified at the end of the manipulations
are those that are present in the tester and absent (or
reduced) in the driver. These molecules are easily
5   isolated after the last PCR step.

       The net result of this method is that a population
of gene fragments that are present in one population and
lost or absent in another population is rapidly isolated.
This is exactly what is needed in the gene identification
10   method. These PCR products can be isolated by standard
techniques and be used for further analysis as shown in
Example 2.

                               EXAMPLE 2
15   IDENTIFICATION OF GENES IN HeLa CELLS
              THAT ARE INVOLVED IN *fas* ANTIBODY SENSITIVITY

       The method of the present invention was applied to
HeLa cells treated with anti-Fas antibody in order to
20   identify genes that when knocked-out cause sensitization
of HeLa cells to the action of anti-Fas antibodies.
       HeLa cells are derived from a human cervical
carcinoma and were used in the original TKO [Deiss and
Kimchi, 1991]. HeLA cells were used as an exemplar of
25   the method of the present system as they are easily grown
in culture, are easily transfected and respond to anti-
Fas antibody treatment.
       Anti-Fas antibody (Kamiya Biomedical Company,
Seattle, Washington, catalog number: MC-060) is directed
30   against Fas/CD95/Apo-1, a transmembrane receptor that is
known to signal a death response in a variety of cell
types. This antibody is an activating antibody, that is,
the binding of the antibody mimics the effects of binding
of ligand. Applying the appropriate dose to responding
35   cells has been shown to lead to induction of cells death

                                   -32-

(Deiss et al., 1996). HeLa cells respond to this
treatment.

In this exemplar genes are identified that regulate
the sensitivity of HeLa cells to killing by anti-Fas

5   antibody. Specifically, genes are identified whose loss
sensitizes HeLa cells to anti-Fas treatment.

The outline of the procedure is as follows:

1. HeLa cells were transfected with an anti-sense
cDNA library.

10   2. Cells containing anti-sense expression vectors
were isolated by selection with Hygromycin. Since the
vector contains the Hygromycin resistance marker, the
selection of the transfected cultures with Hygromycin
generated a population of cells which contain the anti-

15   sense expression cassettes.

3. Aliquots of this pool of cells were treated with
anti-Fas antibody under two different experimental
conditions. It should be noted that more conditions
could be screened at the same time.

20   a. Treatment with a sub-lethal dose of anti-
Fas antibody (10 ng/ml). Cells that are super-sensitive
to treatment with anti-Fas antibody were killed whereas
the majority of the population which is resistant to the
treatment proliferated.

25   b. In the second condition, the cells were
treated with a lethal dose of anti-Fas antibody (100
ng/ml). The cells were harvested at 24 hours, before the
majority of cells had been killed. In this case,
applicants were looking for anti-sense events that

30   accelerate the killing associated with anti-Fas treatment
as another type of sensitization.

4. Aliquots of the cells just before the treatment
with anti-Fas antibody and just after the treatment with
anti-Fas antibody were harvested. The DNA contained in

35   each cell population was extracted.

5. The anti-sense cDNA inserts contained in these DNA samples were preferentially amplified through the use of PCR (see details below).

6. The pools of anti-sense cDNA fragments that were
5 derived from cells after treatment were subtracted from those before treatment (see details below). This generated a set of cDNA fragments that were present in cells before treatment but were absent after treatment. These fragments are good candidates for sensitizing cDNA
10 fragments. In other words, it is likely that expression of some of these fragments leads to the inactivation of genes which causes cells to become super-sensitive to anti-Fas antibody treatment. These super-sensitive cells are quickly killed at a lower dose of anti-Fas antibody
15 or more rapidly than the majority of cells. These cells are therefore lost from the treated cultures but are present in the untreated population. Likewise, the plasmids inducing this super-sensitivity are present in the cells before treatment but are absent from the cell
20 sample taken after treatment. Thus, these fragments are identified during the subtraction.

7. The cDNA fragments generated by the subtraction were cloned into the original expression vector. Appropriate restriction enzyme sites were generated or
25 maintained during the subtraction procedure so that the recloned construct is exactly identical to the construct in the originally transfected cells. The sequence of the isolated cDNA fragments was determined.

8. The anti-sense expression plasmids containing
30 the cDNA inserts that were identified in the method of the present invention were individually re-transfected into HeLa cells and the transfectant cells were assayed for sensitivity to anti-Fas antibody treatment.

35

Specific Materials and Methods

HeLa cells were transfected with anti-sense cDNA
library cloned in the episomal vector, anti-sense
expression vector pTKO-1. This is the same library
described in Deiss and Kimchi [1991]. One million cells
5　　plated in a 100 mm dish were transfected with 15 μg of
DNA containing the anti-sense cDNA library, by using the
Superfect reagent (Qiagen, Santa Clarita, California) as
suggested by the manufacturer. Two days following
transfection, cells were treated with Hygromycin B (200
10　　μg/ml) (Calbiochem-Novabiochem Corporation, La Jolla,
California). Following two weeks of selection, the
population of cells was completely resistant to
Hygromycin B.

These cells were plated in triplicate at a density
15　　of 2.5×10^6 cells per 150 mm dish in the absence of
Hygromycin B. One plate was treated with anti-Fas
antibody at 10 ng/ml (clone CHI-11 Kamiya Biomedical
Company, Seattle, Washington) for five days, the second
plate was treated with 100 ng/ml of anti-as antibody for
20　　24 hours and the third plate was UN-treated for 24 hours.
Following the treatments, the cells were harvested by
washing twice with ice cold PBS (NaCl 8g/liter; KCl
0.2g/liter; Na^2HPO^4 1.44 g/liter; KH^2PO^4 0.24 g/liter;
25　　final pH of solution adjusted to pH 7.4 with HCl) and
concentrated by centrifugation (15,000 x g for 15
seconds). DNA was extracted by using solutions P1, P2
and P3 from the Qiagen Plasmid Purification Kit (Qiagen,
Santa Clarita, California). The cell pellet was
30　　resuspended in 200 μl of solution P1 (50 mM Tris-HCl, pH
8.0; 10 mM EDTA; 100 μg/ml RNase A) then mixed with 200
μl of solution P2 (200 mM NaOH, 1% SDS) and incubated
five minutes at room temperature. 200μl of solution P3
(3.0M Potassium Acetate, pH 5.0) were added and incubated
35　　two minutes at room temperature, followed by a ten minute

centrifugation at 15,000 x g. The clear supernatant was
mixed with an equal volume of isopropanol and centrifuged
at 15,000 x g for ten minutes. The precipitated DNA was
resuspended in 100 μl of water and stored frozen until

5　　use.

For PCT amplification of the cDNA inserts contained
in these DNA preparations, the following reaction was set
in a total volume of 100 μl: 1 μl of the DNA, 200 μM of
dATP, dGTP, dCTP, dTTP, 500 ng each of primers prLPD#64

10　　(SEQ ID No:2) and prLPD#65 (SEQ ID No:3); 10 mM Tris-HCl
pH 9.0; 0.1% Triton X-100; 1.0 mM MgCl and 1 unit of Taq
DNA polymerase (Gibco/BRL, Gaithersburg, Maryland). This
reaction was incubated in a Thermocycler 2400 (Perkin-
Elmer, Foster City, California) according to the

15　　following protocol: First, the reaction was heated to
94°C for five minutes, then was cycled 25 times using the
following three temperatures: 58°C for one minute, 72°C
for five minutes, 94°C for one minute. After 25 cycles,
the reaction was incubated at 72°C for seven minutes.

20　　This resulted in amplification of the cDNA inserts. The
prLPD#64 and prLPD#65 primers were design such that the
end of the cDNA insert that is proximal to the promoter
in the pTKO-1 vector is exactly flanked by a HindIII
restriction site (this site is present in the vector) and

25　　the end of the cDNA that is distal to the promoter in
pTKO-1 vector contains a BamHI restriction site. The
BamHI site was created by altering a single base in the
sequence immediately adjacent to the distal cDNA insert
site (prLPD#65), by PCR. When the library was generated

30　　[Deiss and Kimchi, 1991], this site distal to the
promoter was generated by the fusion of a BamHI
restriction site (derived from the cDNA fragments) and a
BgIII site (derived from the vector). This fused site is
resistant to cleavage by either enzymes, but a single

35　　base change restored the cleavage by BamHI. Thus, the

amplified cDNA fragments are flanked by a HindIII
restriction site on the promoter proximal side of the
cDNA and by a BamHI site on the promoter distal side.
This allows the exact re-cloning of the fragments into
5   the pTKO-1 expression vector with exact conservation of
sequence and orientation.

      Following the PCR reaction, the mixture was cleaved
with BamHI and HindIII (Gibco/BRL, Gaithersburg,
Maryland) as described by the manufacturer. The
10   digestion products were purified using the Wizard PCR
Prep Kit (Promega, Madison, Wisconsin). This generated
cDNA inserts with HindIII and BamHI ends.

      These nucleic acid fragments were subjected to
subtraction using the PCR-Select Kit (Clontech, Palo
15   Alto, California) according to the instructions of the
manufacturer with the following modifications. The
driver was the PCR products derived from the untreated
samples and two testers were used. The first tester was
derived from cells treated with 10 ng/ml anti-Fas
20   antibody and the second tester was derived from cells
treated with 100 ng/ml of anti-Fas antibody. First
modification: the subtraction is done between dsDNA pools
so no cDNA synthesis is required. The fragments
generated from the previous step were used directly in
25   the subtraction. Thus, applicants began at Step IV F3 in
the instructions (preparation of the adapter ligated
tester cDNA). The second modification was the
replacement of the blunt end ligation of adapter 1 and
adapter 2R with cohesive end adapters. These cohesive
30   end adapters were ligated to the BamHI and HindIII
cleaved PCR fragments generated in the step above. The
cohesive ligation is usually more efficient than blunt
end ligation and since applicants use cDNA flanked by
different restriction sites allowing the orientation of
35   the fragments to be maintained when recloning the

subtracted products. If the blunt end ligation is used,
it would not allow distinguishing one end from the other
and applicants would not be able to determine the
relative orientation of the cDNA in the original

5   expression cassette. Thus, adapter 1 was replaced by an
equal mixture of primers prLPD#80 (SEQ ID No:4), prLPD#81
(SEQ ID No:5), prLPD#83 (SEQ ID no:7) and prLPD#84 (SEQ
ID No:8). Adapter 2R was replaced by an equal mixture of
prLDP#82 (SEQ ID No:6), prLPD#88 (SEQ ID No:12), prLPD#89

10   (SEQ ID No:13) and prLPD#90 (SEQ ID No:14). The other
primers were of identical sequence as described in the
kit. Thus, primer prLPD#85 (SEQ ID No:9) is the sequence
of PCR primer 1, primer prLPD#86 (SEQ ID No:10) is the
sequence of nested PCR primer 1 and primer prLPD#87 (SEQ

15   ID No:11) is the sequence of nested PCR primer 2R. The
manual supplied by the manufacturer with the kit was
followed from the point of ligation of the adapters to
the tester (Section IV F3 in the Manual). 0.3 μg of the
tester was taken for adapter ligation. The initial

20   hybridization included 0.9 μg of the driver and 0.03 μg
of the adapted ligated tester. At the conclusion of the
subtraction, a final PCR reaction is done using nested
PCR primer 1 (prLPD#86) and nested PCR primer 2R
(prLPD#87). This material contains the cDNA fragments

25   that were present in the untreated sample but absent from
the treated samples. The product of this PCR reaction
were re-cloned into the anti-sense expression vector.
(Primers used in this example are set forth in Table 1.)
        Re-cloning of the subtracted fragments was

30   accomplished by cleaving the subtracted population with
BamHI and HindIII and purifying the cleaved products with
the Wizard PCR Prep Kit (Promega Madison, Wisconsin).
The cleaved products were then directly cloned into the
pTKO1-DHFR vector between the HindIII and BgIII sites.

35   This replaced the DHFR sequences with the cDNA. This is

precisely the procedure that was used to generate the
anti-sense cDNA expression library. Thus, the fragments
that were generated by the subtraction were exactly re-
cloned into the original anti-sense expression vector

5   that was used to transfect cells at the beginning of the
procedure. The re-cloned constructs exactly duplicate
the constructs that were present in the library. The re-
cloned constructs were introduced into bacteria and DNA
was extracted from the bacteria following conventional

10   methods. These DNA preparations were used as a template
for sequencing in order to determine the nucleotide
sequence of the isolated cDNA inserts. Primer prLPD#51
(SEQ ID No:1) was used in Automated sequencing using
Applied Biosystems 377XL. DNA sequencer with Perkin-

15   Elmer Dye Terminated Sequencing Kits (Perkin-Elmer,
Applied Biosystems Division, Foster City, California).
In addition, plasmids carrying the re-cloned inserts were
transfected into HeLa cells to confirm their ability to
induced super-sensitization to anti-Fas antibody

20   treatment in HeLa cells.

        HeLa cells were transfected with 15 μg of plasmids
or control vectors as described for transfection of the
original library. The cells were selected for two weeks
for resistance to Hygromycin B treatment (200 μg/ml).

25   This selects for cells which contain expression
cassettes. One million cells were plated in a 100 mm
dish and treated with anti-Fas antibody. Effects of
anti-Fas antibody on the transfected cultures were
quantified by MTT assays as described by the manufacturer

30   (Sigma, St. Louis, Missouri)

ANALYSIS OF THE ISOLATED DNA SEQUENCES
        The clones were sequenced using primer prLPD#51
which anneals close to the edge of the cDNA which is

35   distal to the promoter in the antisense expression

cassette.   Thus, in the case that the sequence matches
the sense strand of a known gene then the insert is in
the antisense orientation.   Sequences were compared to
the combined nonredundant database and the dbest compiled
5   at the NCB1 using the Blastn program with default
parameters (Internet address:
http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-
blast?Jform=0).   The sequences determined by the method
of the present invention are listed in Table 2.
10

        Clone LPD#599 (SEQ ID No:15) shows no match against
known gene sequences in the nonredundant database as of
November 9, 1997, but does match several EST sequences
such as a 99% match against gene bank entry AA043612.
15   Sequence analysis indicates that this fragment is
oriented in the sense orientation in the antisense
expression library.   Applicants have noticed that
although the library is designed to preferentially
express the antisense strand, there are some sense gene
20   fragments included in the library [Levy-Strumpf et al.,
1997].
        Clone LPD#601 (SEQ ID No:16) shows no match against
known genes in the nonredundant database as of November
7, 1997, but matches many genomic clone pieces and many
25   EST entries as for example a 95% match to a portion of
gene bank entry N20920.
        Clone LPD#602 (SEQ ID No:17) shows no match against
known genes in the nonredundant database as of November
7, 1997.   It does show some similarity to a large number
30   of gene back entries such as gene bank entry Z68269.
Many of these matches are in the 60-70% range and may
indicate a repeated sequence.
        Clone LPD#606 (SEQ ID No:18) shows no match against
known genes in the nonredundant database as of November
35   7, 1997.   It does shown some matches against mouse EST in

the 80% range (gene bank entry W71379, W29410 and AA409950) and a stretch of a good match against a human EST (gene bank W19764).

Clone LPD#607 (SEQ ID No:19) shows no match against
5   known genes in the nonredundant database as of November 7, 1997. This clone does show a very good match against three EST (gene bank entries, T08248, H42827 and T30569). The sequence analysis indicates that this fragment was transcribed in the antisense orientation in the original
10 library. Thus, reduction (inactivation) of the gene product that is encoded by the full length message representing this clone leads to supersensitization of cells to the treatment with anti-Fas antibodies.

CloneS LPD#608 and LPD#618 (SEQ ID No:20, SEQ ID
15 No:26) show no match against known genes in the combined nonredundant database as of November 7, 1997. They do shown a match with a large number of EST entries (for example, gene bank entry, AA335297, H14907 and AA009451). The sequence analysis indicates that this fragment was
20. transcribed in the antisense orientation in the original library. Thus, reduction of the gene product that is encoded by the full length message representing this clone leads to supersensitization of cells to the treatment with anti-Fas antibodies.

25 Clone LPD#609 (SEQ ID No:21) shows no good match against know genes or EST in the combined nonredundant database as of November 7, 1997.

Clone LPD#610 (SEQ ID No:22) shows no good match against known genes in the combined nonredundant database
30 as of November 7, 1997, but does show a good match against several EST entries including gene bank entries AA447349, H24439, R72995, H17221 and R24985. Sequence analysis indicates that this fragment was in the sense orientation in the original library.

Clone LPD#611 (SEQ ID No:23) shows no good match
against known genes in the combined nonredundant database
as of November 7, 1997, but does show good matches with a
variety of EST (including gene bank entries R76164,

5   R25241, N66591 and N66577). The sequence analysis
indicates that this fragment was transcribed in the
antisense orientation in the original library. Thus,
reduction of the gene product that is encoded by the full
length message representing this clone leads to

10   supersensitization of cells to the treatment with anti-
Fas antibodies.

        Clone LPD#613 (SEQ ID No:24) shows no good match
against known genes in the combined nonredundant database
as of November 7, 1997, but a portion of the sequence

15   shows homology to a large number of sequences and likely
contains a repetitive element.

        Clone LPD#616 (SEQ ID No:25) shows an excellent
match with human tryptophanyl-tRNA synthetase (see emb
X67928 for example). The sequence analysis indicates

20   that this fragment was transcribed in the antisense
orientation in the original library. Thus, reduction of
tryptophany-tRNA synthetase leads to supersensitization
of cells to the treatment with anti-Fas antibodies.

        Clone LPD#619 (SEQ ID No:27) shows no good match

25.   against known genes in the combined nonredundant database
as of November 7, 1997, but does show good matches with
the sequence of a human retroviral element called pHE.1
(for example emb Z95333, emb Z84475, gb M85205).

        Clone L7_10_1_LPD (SEQ ID No:28) shows no good match

30   against known genes in the combined nonredundant database
as of November 7, 1997, but does show very good matches
against several EST (for example, see gb T77711, gb
T78724, gb AA324254). Sequence analysis indicates that
the fragment inserted in the expression library was in

35   the sense orientation.

Clone L7_10_2_LPD (SEQ ID No:29) shows no good match against known genes in the combined nonredundant database as of November 7, 1997, but does show good matches with several EST (for example, see gb R12242 and gb H84498).

5   Sequence analysis indicates that the fragment inserted in the expression library was in the sense orientation.

Clone E7_100_11_LPD (SEQ ID No:30) shows no good match against known genes or EST in the combined nonredundant database as of November 7, 1997.

10   Clone L7_10_8_BS (SEQ ID No:31) shows no good match against known genes or EST in the combined nonredundant database as of November 7, 1997.

Clone L7_10_3 (SEQ ID No:32) shows no good match against known genes or EST in the combined nonredundant

15   database as of November 7, 1997.

Clone E7_100_10 (SEQ ID No:33) shows a good match with mitochondrial DNA (see gb L00016).

Clone L7_100_4_BS and L7_100_5_BS (SEQ ID Nos:34-35) show no good match against known genes in the combined

20   nonredundant database as of November 7, 1997. They do show good matches against several EST entries (for example, see gb AA227149, gb N46661, dbj C76104). Sequences analysis indicates that this gene fragment was oriented in the sense orientation in the original

25   expression cassette.

Clone E7_10_9 (SEQ ID No:36) shows no good match against known genes in the combined nonredundant database as of November 7, 1997. It does show good matches against two EST entries (see gb R54192, gb H39863). The

30   sequence analysis indicates that this fragment was transcribed in the antisense orientation in the original library. Thus, reduction of this protein leads to supersensitization of cells to the treatment with anti-Fas antibodies.

As described above, the isolated fragments were
recloned and then reassayed for sensitivity to treatment
with anti-Fas antibody (50 ng/ml for 72 hrs) using the
MTT assay. These assays showed that expression of

5   fragments LPD#599 (repeated in triplicate with two
different transfectants) or LPD#606 (repeated in
triplicate) resulted respectively in a 1.6 and 2.0 fold
increase in sensitivity to anti-Fas antibody treatment
whereas expression of CrmA (a protective protein)

10   resulted in a 2.3 fold reduction in sensitivity. This
demonstrates that the method of the present invention can
be successfully used to identify genes based on a
positively selected phenotype.

Throughout this application, various

15   publications, including United States patents, are
referenced by author and year and patents by number.
Full citations for the publications are listed below.
The disclosures of these publications and patents in
their entireties are hereby incorporated by reference

20   into this application in order to more fully describe the
state of the art to which this invention pertains.

The invention has been described in an illustrative
manner, and it is to be understood that the terminology
which has been used is intended to be in the nature of

25   words of description rather than of limitation.

Obviously, many modifications and variations of the
present invention are possible in light of the above
teachings. It is, therefore, to be understood that
within the scope of the appended claims, the invention

30   may be practiced otherwise than as specifically
described.

TABLE 1

SEQ ID No:1 prLPD#51 SEQUENCE: TCTGTAGGTAGTTTGTC

SEQ ID No:2 prLPD#64 SEQUENCE: TGGAGGCCTAGGCTTTTGC

SEQ ID No:3 prLPD#65 SEQUENCE: GTAAGGTTCCTTCACAAGGATCC

SEQ ID No:4 prLpd#80 SEQUENCE: CTAATACGACTCACTATAGGGC
TCGAGCGGCCGCCCGGGCAGGTA

SEQ ID No:5 prLPD#81 SEQUENCE: CTAATACGACTCACTATAG
GGCTCGAGCGGCCGCCCGGGCAGGTG

SEQ ID No:6 prLPD#82 SEQUENCE: CTAATACGACTCACTATAGG
GCAGCGTGGTCGCGGCCGAGGTG

SEQ ID No:7 prLpd#83 SEQUENCE: AGCTTACCTGCCCGG

SEQ ID No:8 prLPD#84 SEQUENCE: GATCCACCTGCCCGG

SEQ ID No:9 prLPD#85 SEQUENCE: CTAATACGACTCACTATAGGGC

SEQ ID No:10 prLpd#86 SEQUENCE: TCGAGCGGCCGCCCGGGCAGGT

SEQ ID No:11 prLPD#87 SEQUENCE: AGCGTGGTCGCGGCCGAGGT

SEQ ID No:12 prLPD#88 SEQUENCE: AGCTTACCTCGGCCG

SEQ ID No:13 prLPD#89 SEQUENCE: GATCCACCTCGGCCG

SEQ ID No:14 prLPD#90 SEQUENCE: CTAATACGACTCACTAT
AGGGCAGCGTGGTCGCGGCCGAGGTA

TABLE 2

SEQUENCES

SEQ ID No:15   seqLPD#599pr51 (N indicates uncertainty in sequence)

GATCCAAGCGAAAAAGGGAATAAGAAGGGAGGAATGTAACTAGGAGCAGCTCCCAAC
AGTTTGCCTATGTATTTGCCAGCACCAAAATTTGTAGAGTAAGCCACTTACATTTCC
ACTGCTAGTATTAAGGAAAGACAGCAGTGGTGATTTTTATAAAGCGAGTATACATTT
ATTTTTATTCTGATATGTGAATTTTTCTTTCACGAGTTAATTAACTGGTAATTTGTA
AACAGTGGGAAGAAGATTAGAACAATTATGGAGGTACTGAATTACACAAGGAGATTA
AAATGAAATGAATCAATCTACCTATCTTGTGGTTAGTTAATATTTACCATGATGCAT
ACACTTGAGAAATGAGAAATGCCCAAATTGTATAATGCATTATCTTNNTTATTATTT
ATTNNNGTAAATAATTCTTGCTNNACATTANNCTNCAAGGNTAGCTNTATCTATACT
TGNTAGCTANNTTCTTATACAAAGCANGNTNCTTTTGAANATGATTTACCNATTAAN
TANANNAGCTTAGGTGCCTNNTTTNACTCTGGNTNGTCATNTTGNTTNNCTTTTNNC
NANATCATATATAATTTCCANNGAATGTTGATNTNTNTCCTCANNTTNCATTNTANC
CNGNGCNTATCCTNTNNNNTGNTNGNNTATGTC

SEQ ID No:16   seqLPD#601pr51

GATCCTGGTATAATGTGATTGAATTATGAAGGCAAATTTCTCATGAATGGTTCAGCA
CCATCCCCTTGTACCATCCTCACAATAATGAGTAACTTCTCATGAGATGTAGTCACT
GAAATCTCTATATCACCTCCCCACTCTCCGTGTTTTCTCCTTGCCATGTGAGACAAT
TGATTCTTTCTTTGCCTTCCATGATTATTGAAAGATTTCTGAGGCCTAGAAGCAGAA
GCACTGTGCTTAGAACCATGAGCCAATTAAACCTCTTTTTCAAAATAAATCATACGG
GAAATGGCAAATGAGGACTGGAGCGTTGCTATAAAGATACCTGGAAATGTGGAAGCA
GCTTTGGAACCAGGTAATGGACGGAGGTTGGAAGAGTTTGGAGGGCTCAAAAGAAGA
CAGATGAGAAAACTTTTGGACCATCTTAGAGTCTGGTTCAATGGTTGTGACAAAAAT
CCTGACAGAAACATGGACAGTGAAGGCCAGGCTGAGGAGGTCTCAGAGAGAAATAAG
AAGCTTTTTGCAA

SEQ ID No:17   seqLPD#602pr51

AAACATTATGCACAGAGGAATAAAATTAAGAATGACCACAGTCTTCTCTTCAGAAAT
TATGCAAGCCAGAATAGAGTAGAGCAACATCTTTACAGTTCTTAAAGAAAAAATATA
TCAACTTAGAATTCTATACCCAGCAAAAATATATTTCAAAAAGAAGACACAATACTT
TTTCAGACAGACAAAAGCCAAGAGAGTAATTCCAGGGACGTGTAATATAAGAAATAT
TAGTGAAAGTAGTGTAAAGAATGAGAGAGAGGAAATATAAGGATACTGTTATAATAT
CCCTACACTGTACCTTAAGTGGATTGTAATATTATCTGAAGGTAAACTGTAATATGT
TAAATAAAGATGTATATTTTTAATCCTAGAGAAACTGCTATGAAAACAAAAAGTAAA

ACAAAGTGGTATTACTAACAAGCCAATAATGGAGATAAAATGGGCAATAGAAAACAA
CAACAAAAATTCAATCCAAAAGAAGGAAG

SEQ ID No:18   seqLPD#606pr51

GATCCCTCTGGCACTGGGATGCTTCCACAGATGTACCTGAAAAGTCGTCACTCTCTC
ACCAAGGAGGAAGAAGCAGTACTTTTTGTCTCATAGCATTAGTAACCAAGCTAATG
TTCACCAGTCTGTCATTAGCTCCTGGCTCAGCACTGATCCTGCAAAAGACCGAATTG
AAATCACAAGCT

SEQ ID No:19   seqLPD#607pr51

GGGACATGGTTGGGCCATGCCACACCAGGGCTGGTGAGGCAACCAGTTTTGATTTTG
ACAGAGTGGCTGGAGGAAAAGTGGCAATCAAGGTGCTGCTTGGTTTGCTCTGAGTGC
AAATGGAACCAACAGGTTTCTGCTGCAATCTGTGTGTTCCCAGTGCCAGGTCACACC
AGGAGGGGTGGGGCAGGGCTAACCAAGTGGTCTCTGAACTCACCGAGCGTCTGCACT
TGGTTGTGAAGTTAATGGGAGTACAGAGAGCGTCTGGCCTTGGAGAGGGGTTGAGAG
CCTCCTTTTTGGTTCTTCATTCCTGAGCTCTTGCCTGCCCACAAATCTGACCTCTTT
GAATGGGGACGCAGTCCTTCAACAGAGAAGTTTCTATGGCAAAGAAGTTTCTATTTA
GCTCTAGATCCAGCAGAGTCATCCATTCTAACTGCCCTGAAGTCTAGAGCAGGGGAG
GGAACCCAGAGGCTGGGGATGAGACTAGGCAGACCCTGGTTACCATATGGACAAGGA
CAGGGGAAAGCACCCCCTTCCTCAATTTCTGAAAGTTCTATCTTTGGGTTCGCTGGA
CTTTGAGGATGATAAAGAACATNTAGGTACTA

SEQ ID No:20   seqLPD#608pr51

AAGTGGGACTCTGGGCCTCTGACCAGCTGTGCGGCATGGGCTAAGTCACTCTGCCCT
TCGGAGCCTCTGGAAGCT

SEQ ID No:21   seqLPD#609pr51

GATCCTANACCTGCTCTTTCTCTAATCTTCTGTATCTTAGCAATCGGCCAAAAAACT
TGAAATCATACTTGATTTCTCCTGGCCCTCAAAGCCTACATTCATTCTGCCAAATCA
TGTAAGTCCATCTTTAAAATTTATACTGACTGTGCATTTCTTCTTATCTCCATTACT
ATCACCCAAGTTCAAAATCCTTTCTTATTTTTCACCTGAGTATTGCAATATTTTCTT
AGATGACCCTAACTGATCTTGCCGTTTCTACTCTTGCTTTCCTACAGTCAATTCTCT
TCATATATGCCAACATTACCTTTTAAAACTACAAACAAGTTCAGGTTACTTCTTCCT
TGCTCAAAGTCTCAAATATCTTTTTATCACACACAAATCAAAGGTGGCTAAGAATGG
TCTGGGTCCTGCATACCTCTCCAATGTCATCTACAGCATCTCTGACTTTCTCGCACT
GCTCTGGTCACAGTGGTCTTCTTTTTCTCTAATACACTAAG

SEQ ID No:22   seqLPD#610pr51

TCCAGAGCAAACATTACATCATAGGCCATGGGGGTTGTCACTTAGCTGCATGCTAAG
AATCCTCATATGCTGCTTTGAAAACGCCATAAAAAACTAAGATGCCCTCATACACAG

AAAAAAGGAGTTGGGCCAACAGCCTGCGAGAGAAGATCCTTCCCAACTCTGCCATTC
TTACTGCAGGAGGGTTTGTAATGCTTCCCCACTTCCTCAGGTCCAGCGACTGCACCT
CCACCACTGGAGTGGGGGACACTTCCATTGCTGATGGAAGCTGCCTACTGCTTTTAA
AAACACACACATGAGCTGAGAAGTTCTGCTGAAGGTGGGGAGAGTGCTGCTCTTGGC
TGCCAGCCGGGTCTAGCGGCCCTAACAAGGGAAACTGGCTGATACAAAATGGATCAC
TGCCTGAGATATATGAACTGGACTGAGGATAGAAGTTGCATCCGCTGGGCAAAATGT
GCCAAAGAACAGAAAGTCTTTCAATTTATTCTTTTAGGCACCAAGAATAAATAAATN
CTAAGA

SEQ ID No:23   seqLPD#611pr51

GGGTGAAGCATCTGNTTCATNACACAGGGCTATCATAATTGGGTTCTGGAAATGGAA
TGGGTCTCTAAACATTTTAATGCCCCAGCTTGGTATTGGACNCAATGCAAGATGGCT
AAAATCTTCATTTAAAATTCAAGGGTATTGCTTGCTATTTCACATTATATACTTTTT
AAATACTATTGCTCTTTGCATATGAGGCATTTCACTAACCTTGGATCATTGGATCTT
AAACATGATAATATAGAATATCTGAACATTGGACTTGNTCTTTAGAGTATATGGCCT
TTAGTTCTTGTGGAACTAAATACAGTGATTCTAAGATCAAAAGTCTTAGTGTTTGGG
GTTTTTTTTTCCTCTTTTTGGATATGGGGTGTT

SEQ ID No:24   seqLPD#613pr51

ATCCAGCGGNTCAGCACACACTGGGGCCTACCGGAGTGGGAGGGAGGGACAGGGATG
AGGGAAAAATAACTAATGGGTACTAGGCTTAATACCTGGGTGGTGAAATAATCTGTA
CAACAAACTCCCATGACACAAGTTTACCTTTACAAACCTGCACATGTACCCCTGAAC
TTAAAGAAAAGTTTTTTTAAAAAAATCACACTTCCAGAGTTTGCTAAATAATTATGAC
CAGTATTTTACTACCATCTTCTTCCTTGGTGAACTACTAAAATTAGTAAATTTATGT
TAAAAATGAAGTTCTCCCTAGAAATCAAAAGTGCAGTCTAAGCACTGAAAATGTTCT
ATCAACACTTGTTAACTGAGAACCATTGAAACATCTAGGA

SEQ ID No:25   seqLPD#616pr51

TNCGGATGAGATAGTGAAAGAGTTCATGACTCCCCGGAAGCTGTCCTTCGGCTTTCA
GTAGCACTCGTTTTACATATGCTTATAAAAGAAGTGATGTATCAGTAATGTATCAAT
AATCCCAGCCCAGTCAAAGCACCGCCACCTGTAGGCTTCTGTCTCATGGTAATTACT
GGGCCTGGCCCCTGTAAGCCTGTGTATGTTATCAATACTGTTTCTACCTGTGAGTTC
CATTATTTCTATCTCTTATGGGCAAAGCATTGTGGGTAATTGGTGCTGGCTAACATT
GCATGGTCCGGATAGAGAAGTCCAGCTGTGAGTCTCTCCCCAAAGCAGCCCCACAGT
GGAGCCTTTGGCTGGAAGTCCATGGGCCACCCTGTTCTTGTCCATGGAGGACTCCGA
GGGTTCCAAGTATACTCTTAAGACCCACTCTGTTTAAAAATATATATTCTATGTATG
CGTATATGGAATTGGAAATGTCATTATTGGAACCTAGAAANGGCTTTGGAATATTGA

TGTGGGGAGGNTTATTGAGCACCAGATGTATTTTANCCCATGCCCCCTCCAAAAAGA
AATGGTTAGTNAAAAC

SEQ ID No:26   seqLPD#618pr51

GATCCTTNCAAGTGGGACTCTGGNCCTCTGACCAGCTGTGCGGCATGGGCTAAGTCA
CTCTGCCCTTCGGAGCCTCTGGAAGCT

SEQ ID No:27   seqLPD#619pr51

CTNTGGANTACANCNATCCCTGGATTTAANGAGANGGCCAGGCCACTCTATACTCTA
ATCAAGGAGACCCAGAGGGCAAATACTCATCTAGGAGAATGGGAACCAGAGGCAGAA
ACAGCCTTCAAAACCTTAAA

SEQ ID No:28   L7_10_1_LPD

AATAAGGTTCCTTCACAAANATCCAAGCCAGCACCTTAGTTTTCCTACAACATAAAT
GTAACAAAGTTATCTTCTACTGTATTGCACCTTAGTCCAAAAGTAAAACAACTAAAT
GAAAATTTAAATAAATCAGACTGAAAAAGCCCAAAGAGTAAGAGGAATACCTTATAA
ATGTGACTACCCACCTAAAAATTCTTGAACTACTTTGTTTTGCATAGGATTTTATGG
GACTAACCAAATGTTCCATGAACCATGAGGTGAAAACTGCGATTTCATGATAGCACA
TTGTTTTACAATTCTGATTAGAAATCCTTCAGAAATATTTCTGTATC

SEQ ID No:29   L7_10_2_LPD

ANGTAAGGTTCCTTCACAAAAACCAAGCGGGACTCCNAAACAACCATGGTGCAATGG
TGGCAAAAACTGGGGACTGGGTCTTTCTCGCAACTTCTGTGCCTCCTTTCATTCTCA
AAAAAGGACTATTACCAAATGGGGGGGAAAAAACATAAGCANAAAAAACCCCAAGGA
CAAACTGGAAATTTAATTCCTTNATGCAAATCTTATCTTCATCTGGTGCCTCATTAC
CCTGGGCCCCAAGCCTTCTAATTAGGAAAAAATACTGATTTCTGTTAGGCAATTGCT
TATNTTGGTGGCTTCATCACATTTCA

SEQ ID No:30   E7_100_11_LPD

CAAAGTACCAAGCCCGAGAAAATACAGGTCTATTCAATTCCACCTGCTAGCCTAAAC
TTCAAGTAGACTTCAAAGCAATACAAGTTTGTATGGTCTTTTTGGCAATAGCAGGAA
ATGATACAAGCGAAATCAGTTCTTGATTAAGGGAGGAGCAGAATTGCATTAGTTAGA
TATTCTGGTAGTGCTGCAAACTATAATGATAAATGAAGGCAGCCCAAGACTTAAGAT
GTAAAGTTATGTAGCTCATGCAATTCAATGTCAGTATTTGAAGGCTATATGATGAAT
ATTTCCAGAATTATAATGAAAAAAGTAAAAACAAATTTGCCTCTTACTGATGCTTCA
AAAATCATTTGTGTATATTTAACAAAAGAAGTGTCTAAATAGATCTGAATTTAAACC
ACAGTACTGAACACACTACATGAGGTAACATTGAGTATTATCAAAGTACCAAGCCCG
AGAAAATACAGGTCTATTCAATTCCACCTGCTAGCCTAAACTTCAAGTAGACTTCAA
AGCAATACAAGTTTGTATGGTCTTTTTGGCAATAGCAGGAAATGATACAAGCGAAAT
CAGTTCTTGATTAAGGGAGGAGCAGAATTGCATTAGTTAGATATTCTGGTAGTGCTG

CAAACTATAATGATAAATGAAGGCAGCCCAAGACTTAAGATGTAAAGTTATGTAGCT
CATGCAATTCAATGTCAGTATTTGAAGGCTATATGATGAATATTTCCAGAATTATAA
TGAAAAAGTAAAAACAAATTTGCCTCTTACTGATGCTTCAAAAATCATTTGTGTAT
ATTTAACAAAGAAGTGTCTAAATAGATCTGAATTTAAACCACAGTACTGAACACAC
TACATGAGGTAACATTGAGTATTAT

SEQ ID No:31   L7_10_8_BS

AAGCAAAAGGAATGCTTATACACTGTTGGTAGGAGTATTAGGATTACAGGCGTGAGC
CTCCACTTCCAGCCGTCCACAATCTATTGAGATGACCATGTTGTTTTCTTCCTTCAG
TCTGTTGCACTAATCATTTCCAGCTGATGAAATAACGTTGCATTCCAGGGATACACT
GTACTGGGTACTGGTGTACATTCCTGTTTATATGTTACGGTATTTGGTTTGCCAAAA
TTTACTAGGAAATTTCACAGATCCACTCATAAGCGATATTATCAGAGTTTTCCCTGT
GTATGATGTTTCACTTTGGTATCTAGATAACAATAGCATCATAGAACCAGTAAAAAA
TATTACATGCTCTTCCAAGTTTTTAAGACATTTTTATCGAATTGGTAATTTTCATGT
GTTTGGTATAATATACAAAGAATACATTTGAACTGGGAATTTACATGTGTGTCAGG
GGCAGGGGTGAGAGTTTTTAAATTACCAATTTATTGTCTTCACTTTGTTCTTGAATG
AGTTGTGGTAGTCAGTGTCTTTCTAGGAATTTGTCTACTTCCACAAAGATATTTGTC
AAAATACTAATCCCTTGTTATGGGAA

SEQ ID No:32   L7_10_3

AAGCGGCACTCATCACTTCTACCCTTATTTCTGCTGGCAGAAACCCAGTCACAAGCT
CCACTGACATGCAAGGAGATTTGGGAAATGCAGTCTCTGTCAGCTAGCCCAAACTCT
AAGAAGCCAAAGGAAATATGTATTTTGGGTGGAGATCTAGCCATCTTACCACACTAT
GGTGGTCCAGTAGAGGTCTATCAAAATATTAATTCACAGAAGACTAAAGACACATTT
ACAATGAAGGTTTACAAAACTTATCTGCAAAGAAAAAGCCAGAACATGTTTTATTGT
GGAATAGTTCTAAAATTGCTTATAGATGAAAAGAACAAAACAAATATTTAAATCAGT
CACCTCTAGAATAGTGAAAGGCCAAAAACTGCATTTCAGAAATGAAATATCACTCTG
GGA

SEQ ID No:33   E7_100_10

AAGCGAAAACACCCTCATGTGAATGAGGGTTTTATGTTGTTAATGTGGTGGGTGAGT
GAGCCCCATTGTGTTGTGGTAAATATGTAGAGGGAGTATAGGGCTGTGACTAGTATG
TTGAGTCCTGTAAGTAGGAGAGTGATATTTGATCAGGAGAACGTGGTTACTAGCACA
GAGAGTTCTCCCAGTAGGTTAATAGTGGGGGGTAAGGCGAGGTTAGCGAGGCTTGCT
AGAAGTCATCAAAAGCTATTAGTGGGAGTAGAGTTTGAAGCCCTTGAGAGAGGATT
ATGATGCGACTGTGAGTGCGTTCGTAGTTTGAGTTTGCTAGGCAGAATAGTAATGAG
GATGTAAGTCCGTGGGCGATTATGAGAATGACTGCGCCGGTG

SEQ ID No:34   L7_100_4_BS

TACTGTGATGTTGTTGCTATCTTCATCATCTAACACCTGTGATTTTATATCCATGGT

CACATATGGAAAACCCCCAAGGACAGCCATAACCTCTTCATATTTTTCATCTTCAAG

GAAGTGCAGTAGAGTGTGACGATCTGATTCTTTTAAACTCACCAAATCCTGGATAGT

TTTAATTTTATACTTCTTATGATTAGAAACCCGTCTAAGATTGTCCTCTTCAATATG

AGGGAGCTGCAGAAGGGGAGACTTAAATTGCTGAAGTCCCTGAACGGCCATCTGAGA

SEQ ID No:35   L7_100_5_BS

TACTGTGATGTTGTTGCTATCTTCATCATCTAACACCTGTGATTTTATATCCATGGT

CACATATGGAAAACCCCCAAGGACAGCCATAACCTCTTCATATTTTTCATCTTCAAG

GAAGTGCAGTAGAGTGTGACGATCTGATTCTTTTAAACTCACCAAATCCTGGATAGT

TTTAATTTTATACTTCTTATGATTAGAAACCCGTCTAAGATTGTCCTCTTCAATATG

AGGGAGCTGCAGAAGGGGAGACTTAAATTGCTGAAGTCCCTGAACGGCCATCTGAGA

SEQ ID No:36   E7_10_9

AAGCGGACTTTGGAGGGCAGTGTTATTTTCCCAAAGAAAGACGGCCAAGGGCAGAGG

CATGGATTCTTTGCAGAGCACTTCCTTTTGGTTTTTTCAGTACTGTTTCATAGACAGT

GGGCTCACATGTTCCTGATAGTGCTGCAGTTGCTTAGAAAGCATCCCAGTTAATTGC

AGTAATTAGAACTTCTGGAATATGCTAGGGCAGAAGTATGTCAAGTATGTCACATGA

AGAAAATGTGAAATTCAAGAGTAATCCACACGTGAGAAACTAGACAATGTACATTCA

TGTGTTCTCTTGAAAGGAAAGGGAGAGCTGTAAGCTATCGATACCGTC

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CLAIMS

What is claimed is:

1.   A method for the identification of genes that are essential for the maintenance of specific cell phenotypes including the steps of:

a) identifying a cell type with a phenotype of interest;

b) inactivating genes in the cell type of interest with a gene inactivation means on an aliquot of a culture of the cell type;

c) applying positive selection means to an aliquot of the cell culture of step b;

d) observing cells which continue to maintain the phenotype following gene inactivation whereas cells in which genes necessary for maintaining the phenotype have been lost; and

d) utilizing subtraction analysis means to identify the gene in the cells which has been inactivated that affects the phenotype of interest.

2.   The method as set forth in claim 1 wherein said gene inactivation means are performed using a Genetic Suppressor Element (GSE) inactivation, a Random Homozygous Knock-Out (RHKO) inactivation, or a Technical Knock Out (TKO) inactivation.

3.   The method as set forth in claim 1 wherein the phenotype of interest can be selected from the group consisting of phenotypes relating to growth, phenotypes relating to release of factors and phenotypes relating to cell functions.

4.   The method as set forth in claim 1 wherein the positive selection means can be selected from the group

consisting of ability of cells to survive under specific
culture conditions, ability to express a specific factor,
changes in cell structure, and differential gene
expression.

5. The method as set forth in claim 1 wherein the
subtraction analysis means are selected among the methods
of differential display, representational differential
analysis (RDA), suppressive subtraction hybridization
(SSH), serial analysis of gene expression (SAGE), gene
expression microarray (GEM), nucleic acid chip
technology, or direct sequencing.

6. A gene identified by the method of claim 1.

7. An antibody raised against a gene product whose
sequence is set forth in the gene set forth in claim 6.

8. A method for the identification of genes that
are essential for the maintenance of specific cell
phenotypes including the steps of:

   a) identifying a cell type with a phenotype of
interest;

   b) preparing an expression cDNA library
constructed for the cells as they are expressing the
phenotype;

   c) transfecting a cell culture of the cell type
with an anti-sense expression vectors incorporating the
expression cDNA library;

   d) applying positive selection means to an
aliquot of the transfected cell culture and reserving an
untreated aliquot;

   e) observing cells which continue to maintain
the phenotype and isolating the antisense expression
vector from the cells maintaining the phenotype;

f) identifying anti-sense expression vectors that are present in the reserved aliquot and not in cells maintaining the phenotype by subtraction means whereby anti-sense expression vectors are identified that have targeted genes that maintain the phenotype.

9. The method as set forth in claim 8 wherein the step of recloning and sequencing the antisense expression vectors that target the genes that maintain the phenotype is performed on the identified antisense expression vectors.

10. A kit customized to provide reagents to perform the method of claim 8 including ⎯ ⎯.

a culture of cells of the requested phenotype; and

an expression cDNA library constructed for the cells as they are expressing the phenotype.

11. A nucleic acid having a sequence such that an antisense construct of the sequence inserted into a cell provides reduction of a gene product and thereby sensitization of the cells to anti-Fas antibody.

12. The nucleic acid as set forth in claim 11 having the sequences set forth in SEQ ID Nos:19,20,23,25,26,36.

13. The nucleic acid as set forth in claim 11 wherein the cells are HeLa cells.

14. A nucleic acid having a sequence as set forth in SEQ ID No:18.

15. A nucleic acid having a sequence as set forth in SEQ ID No:21.

16. A nucleic acid having a sequence as set forth in SEQ ID No:25.

17. A nucleic acid having a sequence as set forth in SEQ ID No:30.

18. A nucleic acid having a sequence as set forth in SEQ ID No:31.

19. A nucleic acid having a sequence as set forth in SEQ ID No:32.

Add
A6

ADD B1